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Brief Communication

# The Kalimantacin/Batumin Biosynthesis Operon Encodes a Self-Resistance Isoform of the FabI Bacterial Target

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## SUMMARY

**BatG is a trans-2-enoyl-ACP reductase, encoded in the kalimantacin/batumin (kal/bat) biosynthesis operon. It is not essential for the production of the kal/bat secondary metabolite. Instead, BatG is an isoform of FabI, conferring full resistance to target bacteria. It also complements FabI in its role in fatty acid biosynthesis. The identification of FabI as the antibacterial target is important to assess clinical potential of the kalimantacin/batumin antibiotics against *Staphylococcus aureus*.**

## INTRODUCTION

Bacteria produce fatty acids through a type II system (FASII). Each reaction is catalyzed by a discrete enzyme. The FASII pathway is essential to cell growth and the trans-2-enoyl reductase is responsible for the final and rate-limiting step in each cycle. As such, this enzyme is essential for the viability of bacteria (Heath and Rock, 1995). Since the initially identified trans-2-enoyl reductase FabI (e.g., *Escherichia coli, S. aureus*), other FabI isozymes were discovered in other species, such as FabL and FabK in *Bacillus subtilis* and *Streptococcus pneumoniae,* respectively (Heath and Rock, 2000; Heath et al., 2000). Recently, reports on yet another FabI isoform (FabV) were published from *Vibrio cholerae* (Massengo-Triassé and Cronan, 2008), *Pseudomonas aeruginosa* (Zhu et al., 2010), and *Burkholderia mallei* (Lu and Tonge, 2010). These isoforms show only weak sequence homology with other trans-2-enoyl reductases.

Given their selectivity for bacteria over mammals and the highly conserved FASII system between species, fatty acid inhibitors have long been undisputedly potent antibacterial compounds (Payne et al., 2002). Several antibiotics target bacterial fatty acid synthesis, three of which (Triclosan, diazaborines, and isoniazid) target the trans-2-enoyl-ACP reductase (FabI) (Heath et al., 2002). However, in recent reports their potential in clinical settings has been hotly debated. Brinster et al. (2009) showed that major Gram-positive pathogens can overcome FASII inhibition through exogenous fatty acid incorporation. On the other hand, others invalidated these results for *S. aureus* and showed that FabI inhibitors do work in vivo (Balemans et al., 2010).

Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a major threat in both hospital and community settings, since it is developing resistance to the so called ''last-line'' antibiotic vancomycin. To combat this emerging antibiotic resistance of pathogenic bacteria, new antibacterial agents are needed. The kalimantacin antibiotics are promising novel antibacterial agents with a strong selective antistaphylococcal activity (0.05 μg/ml) and moderate activity against enterobacteria (1–10 μg/ml) (Kamigiri et al., 1996). The kalimantacin antibiotics were isolated from a fermentation broth of *Alcaligenes sp.* YL-02632S (Kamigiri et al., 1996; Tokunaga et al., 1996). Batumin, a compound with the same gross molecular composition as kalimantacin A, has been isolated from a fermentation broth of *Pseudomonas batumici* (Smirnov et al., 2000). To date, no batumin-resistant clinical *Staphylococcus* isolates were reported (Klochko et al., 2008) and the mechanism of action of these antibacterial compounds has not yet been reported. However, batumin influences cell wall morphology, causing the peripheral wall to differentiate into an outer and inner layer (primary and secondary wall, respectively; Giesbrecht et al., 1998).

Recently, we reported the isolation of kalimantacin/batumin (kal/bat) from *P. fluorescens* strain BCCM_ID9359 and experimentally elucidated its biosynthesis gene cluster (Mattheus et al., 2010). High-resolution MS, $^{1}$H, and $^{13}$C NMR spectra data verified the polyketide structure (Figure 1) as reported for kalimantacinA/batumin (Mattheus et al., 2010). The kal/bat gene cluster consists of 16 open reading frames (ORFs), encoding a collinear hybrid PKS-NRPS system (Bat1-3), extended by *trans*-acting tailoring functions (BatA-M). We here report that BatG encodes a functional FabI isozyme which confers full resistance to kalimantacin/batumin and complements FabI, marking this enzyme as the bacterial target for these antibiotics.

## RESULTS AND DISCUSSION

### Bioinformatical Analysis Reveals BatG as a Predicted Trans-2-Enoyl-ACP Reductase Isozyme

Initial similarity searches revealed BatG similarity to trans-2-enoyl-ACP reductases, involved in the formation of a saturated acyl-ACP by an NAD(P)H-dependent reduction of the trans-2-enoyl-ACP

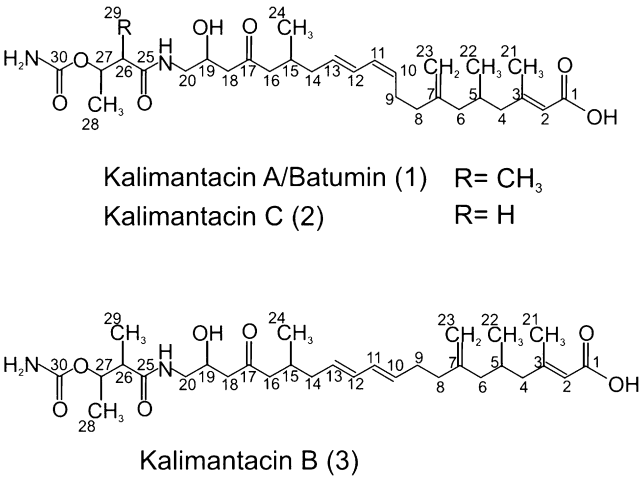


**Figure 1. Structure of Kalimantacins, Batumin, and kal/bat**
The molecules have a linear polyketidal backbone with an incorporated glycine, multiple methyl branches, and a characteristic carbamoyl group.

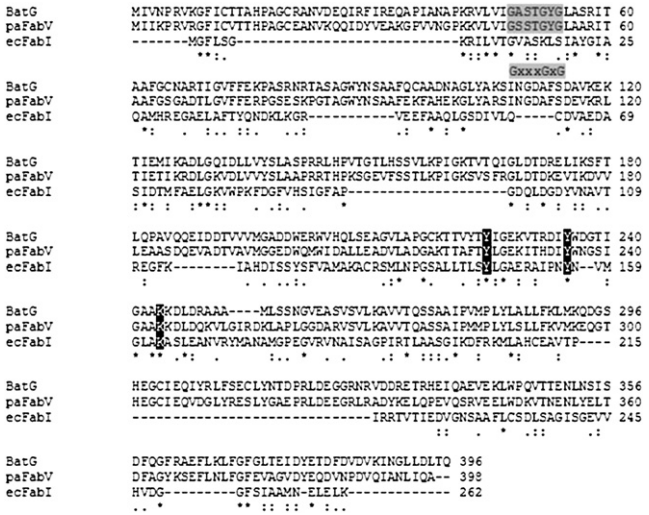


**Figure 2. Multiple Alignment of BatG with ecFabI and paFabV**
BatG shows strong similarity with paFabV and moderate similarity with ecFabI. NAD(P)H binding motif (GxxxGxG) and catalytic residues (Y-Y-K) are highlighted in gray and black, respectively.

double bond (Mattheus et al., 2010; Heath et al., 2002). These enzymes are essential for the final step of the elongation cycle of fatty acid biosynthesis, a reaction catalyzed by FabI in *E. coli* and *S. aureus*. Multiple alignment of BatG with ecFabI and paFabV shows only moderate similarity with ecFabI (as seen with other experimentally proven trans-2-enoyl-ACP reductase isoforms like FabK, FabL), but strong homology to paFabV (59% identity, 76% similarity) (Figure 2). The conserved NADH-binding motif (GxxxGxG) and catalytic triad (Y-Y-K) are present. As in paFabV, BatG contains a Y-$X_8$-K active site motif, rather than the Y-$X_6$-K motif present in ecFabI. Based on this sequence homology, we hypothesize that BatG is a trans-2-enoyl-ACP reductase isozyme.

## BatG Knockout Analysis Does Not Influence the kal/bat Biosynthesis Structurally

To study the role of BatG in the biosynthesis of kal/bat, we generated a specific in vivo gene inactivation by in-frame deletion of *BatG*, minimizing the risk of polar effects. The phenotype of the ORF-specific mutant was examined by plate bioassay, HPLC purification, FT-MS analysis, and $^{1}H$, $^{13}C$ NMR analysis and compared with results from the wild-type strain. An identical HPLC retention time of 20.8 min (see Figure S1A available online) and a fully conserved antibacterial activity on a bacterial lawn plate assay suggest no change in structure or conformation. This is confirmed by an HRMS molecular peak at [M + Na]/e = 571.3339, molecular formula $C_{30}H_{48}N_2O_7Na$ (<1 ppm error), while $^{1}H$ and $^{13}C$ NMR spectra data further verify the wild-type kal/bat structure (1) (Figure 1; Figures S1B–S1D). In addition, the reported inactive 17-hydroxy kal/bat intermediate found in chloroform extracts of wild-type cells is also present in the *BatG* knockout strain. The only noticeable difference between the wild-type strain and the *BatG* knockout strain is the total yield of kal/bat production, 35 and 25 mg/l, respectively. This reduction is not attributed to growth retardation of the *BatG* knockout strain (data not shown). These facts prove that the kal/bat biosynthesis is independent of BatG, despite its localization in the operon among kal/bat tailoring enzymes. In the kal/bat producing strain, *P. fluorescens* BCCM_ID9359, BatG is not the sole resistance determinant since the BatG knockout strain is still fully resistant (Table 1). Basal level shotgun sequencing on strain BCCM ID9359 DNA (as described in Mattheus et al., 2010) indicates the presence of another FabV homolog, not linked to the kal/bat biosynthesis cluster, which may explain the intrinsic kal/bat resistance (data not shown). Our results show that *P. fluorescens* pf5 is resistant to triclosan, yet sensitive to kal/bat. In contrast, *P. aeruginosa* PAOI and *P. fluorescens* BCCM_ID9359 are resistant to both triclosan and kal/bat. The FabV homolog, PA2950, has been shown to be responsible for triclosan resistance in *P. aeruginosa* PAOI (Zhu et al., 2010) and is probably the determinant for kal/bat resistance too. However, *P. fluorescens* pf5 also encodes a FabV homolog (PFL3335). Structural studies revealed that the affinity of triclosan to trans-2-enoyl-ACP reductases is attributable to the interactions with the flexible NADH cofactor-binding loop. The closed, ordered conformation of the loop is a major determinant for the enhanced binding of triclosan (Pidugu et al., 2004). Specific point mutations can change this conformation and result in poor triclosan binding properties (Xu et al., 2008). The differences in kal/bat and triclosan sensitivity of *P. fluorescens* pf5, *P. aeruginosa* PAOI, and *P. fluorescens* BCCM_ID9359 could be explained by a different binding loop conformation in PFL3335. However, PA2950 and PFL_3335 show slightly higher overall homology (89%/79%, similarity/identity) compared to the homology with the FabV homolog in *P. fluorescens* BCCM_ID9359 (77%/60%, similarity/identity) and no specific different regions/residues could be identified.

We have also previously shown that kal/bat is produced as an inactive 17-hydroxy kal/bat precursor, which is activated by oxidation upon export (Mattheus et al., 2010). This efflux-coupled activation may form an additional self-resistance system for *P. fluorescens* BCCM_ID9359 to prevent high intracellular antibiotic concentration during production.

**Table 1. MIC Values**

| Strain | MIC (μg/ml) | | | |
|---|---|---|---|---|
| | Kal/Bat | | | Triclosan |
| | MH + 2% glucose | MH + [0–0.01 mM IPTG] | MH + [0.1–1 mM IPTG] | MH + 0.5 mM IPTG |
| *E. coli* S17-1 | 4 | 2 | 2 | 0.1 |
| *E. coli* S17-1+pJH10 | 1 | 1 | 1 | 0.1 |
| *E. coli* S17-1 +pJH10_BatG | 1 | 1 | >128 | 32 |
| *E. coli* S17-1 +pSK5632_BatG | >128 | >128 | >128 | 32 |
| *P. fluorescens* Pf5 | 2 | 2 | 2 | 64 |
| *P. fluorescens* Pf5 +pJH10_BatG | >128 | >128 | >128 | 64 |
| *P. fluorescens* BCCM_ID9359_ΔBatG | >128 | >128 | >128 | 64 |
| *S. aureus* RN4220 | 0.1 | 0.1 | 0.1 | 0.1 |
| *S. aureus* RN4220 + pSK5632_BatG | >128 | >128 | >128 | 64 |

Minimal inhibitory concentrations were determined using NCCLS standards (National Committee for Clinical Laboratory Standards, document M7-A5). pSK5632: ApR, CmR, thèta replication, Plac. pJH10: TcR, SmR, oriT, Ptac, lacIq.pSK5632 results in constitutive expression in both *E. coli*, *P. fluorescens*, and *S. aureus*. To control the tac-promotor regulated expression of pJH10_BatG in E. coli, glucose (2%) or varying IPTG concentrations [1 μM- 1mM] were added. pJH10 is constitutively expressed in *P. fluorescens*, as shown in other expression experiments (Mattheus et al., 2010).

### Heterologous BatG Expression Provides Complete Resistance to kal/bat in Sensitive Strains and Complements FabI as an Essential Enzyme in Fatty Acid Biosynthesis

Expression of BatG in *trans*, using the broad-host-range IncQ expression vector pJH10 (El-sayed et al., 2001), in both *P. fluorescens* pf5 and *E. coli* S17-1 results in a pronounced increase of the MIC, from 2 μg/ml to complete resistance to kal/bat (MIC > 128 μg/ml) (Table 1). Expression of BatG in *S. aureus* RN4220 (using the low-copy-number shuttle vector pSK5632; Grkovic et al., 2003) also confers complete resistance to batumin (MIC from 0.05 to >128 μg/ml). Variation in BatG expression level by controlled IPTG induction rules out a dose dependent effect, indicating that the resistance is due to the expression of the kal/bat-resistant BatG rather than overexpression of the target. BatG expression also confers resistance to triclosan, another FabI inhibitor, with MIC drastically increasing from 0.064 to 32 μg/ml and 64 μg/ml for *E. coli* and *S. aureus*, respectively (Table 1). This further indicates a common antibacterial target of kal/bat and triclosan.

To test whether BatG can functionally complement FabI, the expression plasmid was introduced into the *E. coli fabI*(Ts) strain JP1111 (Egan and Russell, 1973). This strain is unable to grow at the nonpermissive temperature of 42°C due to the *fabI*392 mutation. JP1111 strains complemented with BatG grow at 42°C, whereas strains carrying the vector do not (Figure 3). Thus, BatG functionally complements the *E. coli fabI* mutation, showing its in vivo trans-2-enoyl-ACP reductase activity.

Correlating these data to the previously described host spectrum of activity of kal/bat (Mattheus et al., 2010) suggests that species which rely solely on FabI homologs for their fatty acid synthesis (*S. aureus, E. coli*) are effectively inhibited, while strains with an alternative trans-2-enoyl-ACP reductase isoforms (*B. subtilis, P. aeruginosa, S. pneumoniae*) are resistant to kal/bat.

### Conclusions

Kal/bat has a strong antistaphylococcal activity with a MIC of 0.05 μg/ml, making it a potent new antibiotic to cope with resistant *S. aureus* (MRSA) infections. The results presented here prove that kal/bat antibacterial activity is caused by FabI trans-2-enoyl-ACP reductase inhibition during fatty acid biosynthesis, a highly relevant and intensely discussed antibacterial target. The trans-2-enoyl-ACP reductase isoform BatG is resistant to both kal/bat and triclosan inhibition. The broad-host-range complementation capability of this natural enzyme (*P. fluorescens*, *E. coli*, and *S. aureus*) suggests a potential and unwanted source of resistance against FabI inhibitors in clinical settings. Hence, these findings are important for future research on FabI as an antibacterial target and for clinical potential of the kalimantacin/batumin-related antibiotics against MRSA.

## SIGNIFICANCE

**To combat the emerging antibiotic resistance of pathogenic bacteria like methicilin-resistant *Staphylococcus aureus* (MRSA, a critical pathogen in hospital environments), new**

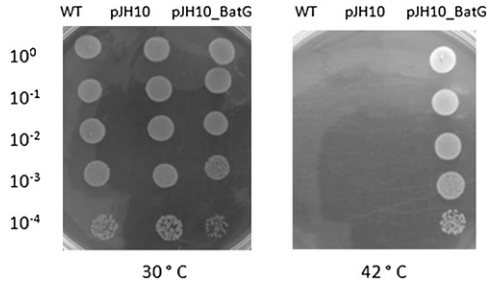


**Figure 3. Growth of *E. coli* JP1111 FabI(Ts) Strain Complemented with BatG**

BatG was cloned into pJH10 low copy number vector under control of the *tac* promotor. Both empty vector (pJH10) and construct (pJH10_BatG) were transformed into FabI(Ts) strain JP1111. Serial 10-fold dilutions of each overnight culture were spotted on plates of LB supplemented with 0.5 mM IPTG and incubated at 30°C (left) and 42°C (right), respectively.

**potential antibacterial agents (like the kalimantacin antibiotics) are being investigated. In this report, we show that the kalimantacin antibiotics inhibit bacterial fatty acid synthesis (FASII pathway) and we describe a natural resistance gene. These results have strong implications for the clinical potential of the kalimantacin antibiotics, since the essential nature of the FASII pathway in Gram-positive pathogens is currently under debate.**

## EXPERIMENTAL PROCEDURES

### Bacterial Strains, Plasmids, and Culture Conditions

*P. fluorescens* strain BCCM_ID9359 was used as the wild-type kal/bat producer. The strain was grown at 28°C in tryptose broth (Merck, Germany) for liquid cultivation and on tryptose agar (Merck) for solid cultivation. *E. coli* Transformax EC100 (Epicenter, US) was used for plasmid preparations, *E. coli* S17-1 was used for conjugal transfer of DNA into *P. fluorescens* strain BCCM_ID9359. *E. coli* strain JP1111 was obtained from the Coli Genetic Stock Center (CGSC, Yale University). Strains were grown at 37°C in lysogeny broth (LB) and LB agar (LB supplemented with 1.5% w/v agar). *S. aureus* ATCC6538 was used in bioassays to monitor kal/bat production. Media were supplemented with one ore more antibiotics at appropriate concentrations: ampicillin (100 mg/l), kanamycin (50 mg/l), triclosan (25 mg/l), tetracycline (15 mg/l). Plasmids used during this work are listed in Table S1.

### Production, Isolation, and Analysis of kal/bat

*P. fluorescens* strain BCCM_ID9359 was seeded in 250 ml tryptose broth in a 1L Erlenmeyer flask and incubated at 16°C on a rotatary shaker (200 rpm) for 48 hr. To isolate kal/bat, the culture was adjusted to pH 10 with NaOH, and the cells removed by centrifugation. After acidification with formic acid to pH 3, the supernatant was extracted with chloroform (2 × 250 ml) and concentrated in vacuo. The extract was either used directly for HPLC analysis or further purified by silica gel column chromatography. The HPLC analysis was carried out on an Alltima C-18 column (5 μm, 250 × 4.6 mm, Alltech). The column was equilibrated with 100% solvent A (5% acetonitrile (ACN), 0.1% TFA) and developed according to the following program: (0–1 min, 100% A; 1–30 min, a linear gradient from 100% A to 100% ACN; 30–40 min, linear gradient from 100% ACN to 100% A) at a flow rate of 1 ml/min and UV detection at 228 nm using a Shimadzu SPD-10A detector.

Silica gel column chromatography (20 × 250 mm; $CH_2Cl_2$/MeOH/HCOOH (100/4/0.1 to 100/10/0.1) was used for large scale purification prior to NMR analysis and microbiological testing. MS analysis was performed on a Bruker Daltonics Apex-Qe FT mass spectrometer and NMR on a Bruker Ultrashield Avance instrument, operating at 600/300 MHz for $^{1}H$ and 150/75 MHz for $^{13}C$ nuclei in $CDCl_3$.

### Targeted Inactivation

Directed, in-frame gene deletion of BatG was achieved by cloning two DNA fragments of approximately 300–500 bp flanking *BatG* into the suicide vector pAKE604 (El-sayed et al., 2001) (pAKE604_BatG; primers are listed in Table S1). DNA transfer to *P. fluorescens* strain BCCM_ID9359 was performed through biparental mating with *E. coli* S17-1. A late exponential culture of *E. coli* harboring the relevant plasmid and *P. fluorescens* strain BCCM_ID9359 were mixed on a 0.45 μm sterile Millipore filter, placed on an LB-agar plate. After overnight incubation at 28°C, the mixture was resuspended in 1 ml of saline solution and spread on LB-agar plates supplemented with plasmid-selective antibiotic and triclosan. Cointegrant clones were picked and incubated in tryptose broth without selection overnight at 30°C. Serial dilutions were spread on tryptose plates containing 5% sucrose, to select for vector excision. Deletion knockouts were screened by replica plating and PCR analysis.

### Heterologous BatG Expression

The BatG open reading frame was amplified and cloned into pJH10 (El-sayed et al., 2001) under control of the *tac* promoter (pJH10_BatG). For BatG, the difference in G+C-content between Gram-positive and Gram-negative bacteria, expression (transcription, translation) of high G+C-content genes in Gram-negative bacteria did not cause a problem: BatG and its upstream region was amplified and cloned into the low-copy-number *S. aureus* shuttle vector pSK5632 (Grkovic et al., 2003) (pSK5632_BatG) (primers are listed in Table S1). DNA transfer to *P. fluorescens* was performed through biparental mating with *E. coli* S17-1. *S. aureus* RN4220 was transformed with pSK5632_BatG by electroporation as described by Schenk and Laddaga (1992). Expression of BatG was induced by varying IPTG concentrations (1 μM to 1 mM) to examine an expression dose-dependent effect.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2010.07.015.

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